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Lithium-containing S-PRG fillers promoted the wound healing process of pulp tissues through activation of Wnt/β-catenin signaling pathway

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Introduction

The main goal of direct pulp capping is to maintain the vitality of the pulp tissue, even when it is exposed due to bacterial invasion, iatrogenic mechanical preparation, or trauma[1]. Mineral trioxide aggregate（MTA）has been considered as a potential gold standard of vital pulp therapy because it showed integrity to the pulp tissues compared with calcium hydroxide[2]. The exact mechanism of MTA to induce dentin regeneration is not clear but they also release calcium ions that show alkalinity to a level lesser than calcium hydroxide and it has the ability to stimulate growth factor secretion by the cells to aid in migration, differentiation and crystalline formation[3]. Although MTA bears some disadvantages（e.g., long setting time, does not bond with dentin, causes tooth discoloration, displays toxicity when freshly mixed, and is difficult to handle）[4,5], its utility and benefits have inspired the dental research community and collaborators to invent new bioactive particles with high reliability. Various bioactive materials have been introduced and tested for their efficacy in dentin regeneration and pulpal healing, such as calcium-silicate–based cements, tetracalcium phosphate cement, and "Giomers"[6,7,8,9]. Giomer refers to any product that contains surface pre-reacted glass（S-PRG）filler particles: stable glass ionomers formed by acid-base reactions of fluoro-boro-alumino-silicate glass with poly-alkenoic acid in a hydrated environment. The reaction occurs when silicate glass-treated surfaces react partially with a poly acrylic acid solution[10,11]. Unlike other bioactive silicate materials that can release calcium ions, these tri-laminar structures of surface pre reacted glass fillers are able to release fluoride ions as well as five other ions（Al^{+3}, BO^{-3}, Na^{+}, SiO_3^{-2} and Sr^{+2}）[12]. They possess antibacterial properties[13,14,15]; can remineralize the dentin, because of the fluoride and silicate ion content[16,17,18]; have acid-buffering capacity[19]; and can prevent plaque accumulation[20]. The strontium and borate ions in these fillers are reported to have bioactive roles in osteoblast differentiation and bone regeneration[21]. These properties increase their importance and utility in the preventive and operative dental fields[19,22]. We previously demonstrated that a prototype pulp-capping cement containing S-PRG fillers without additives like lithium could induce reparative dentin formation in a rat model[23]. Lithium compounds have been first introduced into the clinic in 1970 after being approved by the FDA to treat the acute symptoms of patients with

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Table 1　Compositions of surface pre-reacted glass (S-PRG) fillers and liquids.

Powder	Liquid	Components of the liquid
S-PRG filler (Multifunctional glass fillers)	-	-
	FGL-02	Copolymer of Acrylic acid and Tricarboxylic acid Water, Others, No LiCl (PH < 1)
	FGL-02-LiCl-10 mM	Copolymer of Acrylic acid and Tricarboxylic acid Water, LiCl (10 mM), Others (pH < 1)
	FGL-02-LiCl-100 mM	Copolymer of Acrylic acid and Tricarboxylic acid Water, LiCl (100 mM), Others (pH < 1)
	FGL-02-LiCl-1000 mM	Copolymer of Acrylic acid and Tricarboxylic acid Water, LiCl (1000 mM), Others (pH < 1)
MTA (Tri-calcium silicate, di-calcium silicate, tri-calcium aluminate, bismuth oxide, and calcium sulfate)	Distilled Water	-

bipolar diseases[24]. Subsequent in vitro studies reported that lithium ions can accelerate bone regeneration and upregulate osteoblast differentiation and mineralization[25,26]. Ishimoto et al. revealed that 10 mM LiCl can induce tertiary tubular dentin formation when applied topically to pulpotomized teeth in rats, and confirmed activation of the canonical Wnt/β-catenin pathway when pulp cells were treated with lithium ions in vitro[27]. So, in this study, our hypothesis was that, incorporation of Li ions into a new S-PRG filler would enhance dentin formation through activation of Wnt/β-catenin signals. Therefore, our objective was to develop a new bioactive pulp capping cement using this material. To achieve the goal, we conducted both animal and cell-based studies to tailor an effective S-PRG/LiCl combination that can activate the Wnt/β-catenin signaling pathway.

Methods and Materials:

Materials used in the study as shown in table 1.

1. Animal study

(1) Pulp-capping experiment

This study was conducted following approval from the Animal Care and Use Committee at Osaka University Graduate School of Dentistry (approval No. 28-013-0). Twenty-four-8-week-old male Wistar rats (180-200 g) were used (Japan Animal Inc., Tokyo, Japan). The groups were subdivided into four groups according to the time of pulp harvesting. Thirteen rats for histopathology and eight for immunofluorescence assessment. Rats were anesthetized and pulps were capped as described in a previous study[28]. At 1,7,14 and 28 days postoperatively, the rats were sacrificed, the maxillae dissected, and the teeth prepared for scanning using a micro-CT scanner (R-MCT2, Rigaku, Tokyo, Japan). Then the samples (28 days post-operatively were prepared for histopathological evaluation using hematoxylin and eosin staining (Muto Pure Chemicals, Tokyo, Japan) (n=5 for each group).

(2) Assessment of Wnt/β-Catenin Signaling Pathway by Immunofluorescence Staining

Sixteen rat's pulps had been subdivided to five groups (intact tooth, capped with S-PRG (7 and 14-days) or S-PRG/LiCl-100 mM cements (7 and 14- days) were evaluated using immunofluorescence staining to determine activation of Wnt/β-catenin signaling. Evaluation of signaling markers were conducted against rabbit monoclonal β-catenin (ab32572, Abcam; Cambridge, UK) or rabbit polyclonal Axin-2 (ab32197, Abcam). Images were taken and positive stainings were measured using the BZX-800 view Analyzer Software (Keyence, Osaka, Japan). (n=3) for each group.

(3) Lithium ion levels in rat blood serum

At 24 h post-capping, 5 ml of blood samples were collected from three rats to evaluate the concentration of lithium ions in the serum using the flame method of

atomic absorption spectroscopy (SpectrAA-240, Agilent Technologies; Santa Clara, CA). (n=3)

2. *In Vitro* studies

(1) Compressive and shear bond strength to dentin

In accordance with ISO9917-1, compressive strength of different S-PRG cements and cylindrical samples were used to measure the shear bond strength by universal testing machine.

(2) Cell studies

In vitro cell studies were conducted to identify the optimal concentration of LiCl. Human Dental Pulp Stem Cells –primary cells collected from human dental pulp tissue of wisdom tooth- (hDPSCs; Lonza, Basel, Switzerland). They were cultured in *a*-Minimum Essential Media (*a*-MEM, Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS; Gibco) added into eluate solutions of different cements. Cells between passage 3 and 5 were used in subsequent assays.

To assess the viability, trypan blue staining of the cultured hDPSCs were counted. Cytotoxic activity was conducted using lactate dehydrogenase enzyme activity. Cell migration was evaluated using a wound healing assay. For differentiation and mineralization of hDPSCs, alkaline phosphatase and alizarin red staining assays were used consecutively.

3. Statistical methods

Numerical values of all experimental groups were statistically evaluated using one-way ANOVA with Tukey–Kramer post hoc tests for cell studies and student's-t test for mechanical properties assessment. P-values < 0.05 were considered significant.

Results

1. Pulp capping experiment

Treatment with S-PRG/LiCl-10 mM and S-PRG/LiCl-100 mM led to the formation of complete tertiary dentin structures that were continuous with the primary dentin (PD) without any defects, similar to that produced by MTA, which was used as a positive control. In contrast, S-PRG/LiCl-1000 mM treatment led to incomplete tertiary dentin formation and the S-PRG cement without lithium ions resulted in a defected tertiary dentin structure (Fig.1).

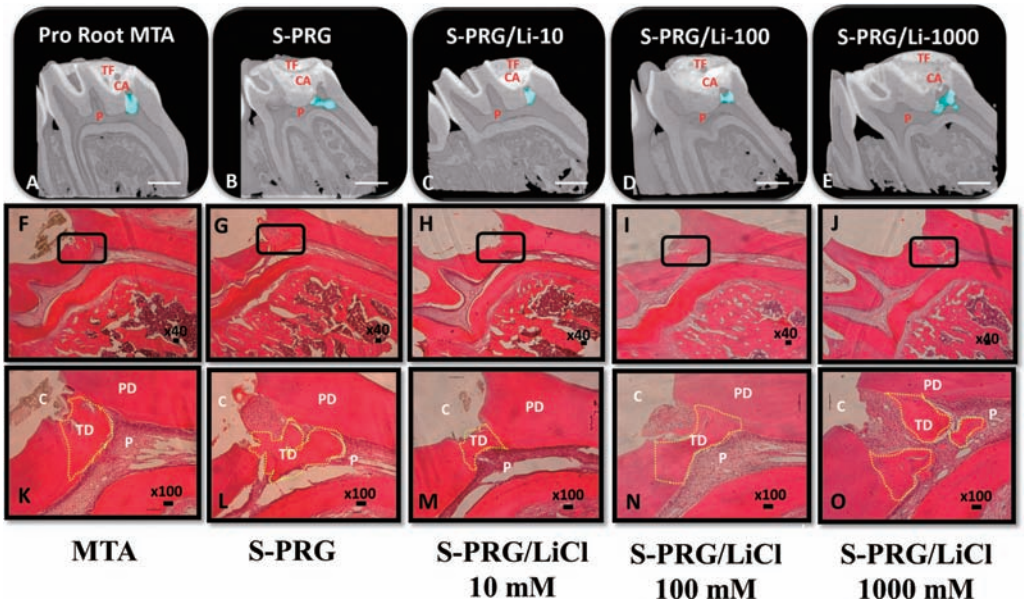

Fig. 1. Micro-CT and histopathological evaluation of different pulp-capping materials. (A-E) CT images analyzed by TRI 3D BON software, displaying the area of tertiary dentin formed in response to different pulp-capping materials (TD in blue) 28-days post capping. (F-J) H&E-stained sections (5-μm-thick). (K-O) H&E-staining at higher magnification. (P: pulp, CA: capping agent, TF: temporary filling, TD: tertiary dentin, PD: primary dentin, C: cavity, Scale bar =100 μm, n=5).

2. *In vitro* studies

(1) Compressive and shear bonding strength

S-PRG/Li-100 mM and S-PRG/Li-1000 mM specimens had significantly decreased the compressive strength compared to other groups. However, there was no significant difference in the shear bond strength of the cements to dentin among any of the groups. (see table 2)

Table 2　Mechanical properties of the cements used in this study.

Cement	S-PRG	S-PRG/ Li- 10mM	S-PRG/ Li- 100mM	S-PRG/ Li-1000 mM
Compressive strength (MPa) n=5	70±7	65±7	52±7*	46±4*
Dentin shear bond strength to dentin (MPa) n=5	1.7±0.3	1.9±0.8	1.9±0.4	1.3±0.2

(Means±SD) in MPa, n=5, One-way ANOVA with t-student test. *P<0.05.

(2) Cell studies

① Proliferation assay

hDPSC survival was maintained in the presence of eluates from S-PRG/LiCl-10 mM and S-PRG/LiCl-100 mM samples when S-PRG/Li-1000 mM decreased the viability. (Fig. 2a)

② Cytotoxicity assay

Cells exposed to S-PRG/LiCl-1000 mM showed higher cytotoxicity as compared with those in the S-PRG/LiCl-100 mM group. (Fig. 2b)

③ Cell migration (wound healing assay)

The S-PRG/LiCl-100 mM showed the best effect on cell migration as compared with the other lithium-containing groups (Fig. 2c).

④ Alkaline phosphatase staining

The intensity of ALP staining was significantly higher in cells that were cultured in the presence of eluates from the S-PRG/LiCl-100 mM group (Fig. 2d).

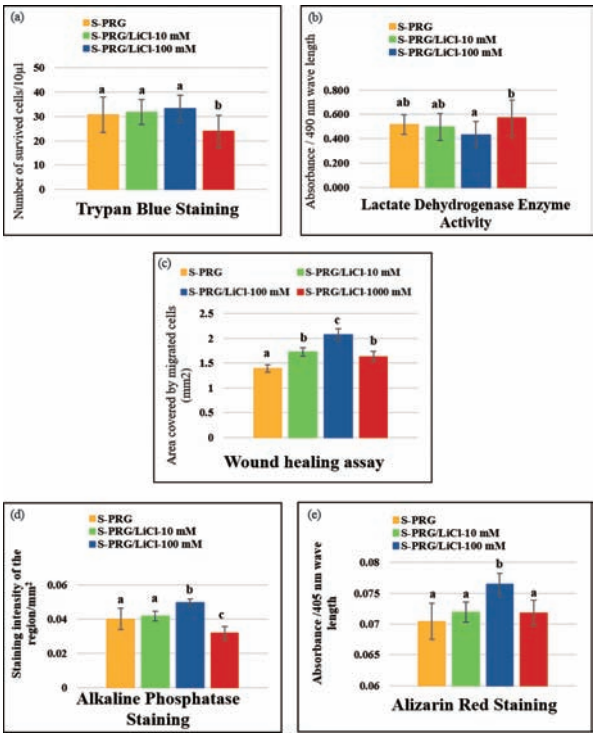

Fig. 2.　*In vitro* cell studies outcomes. The results showing changes in cell phenotype after exposure to eluates from S-PRG cements containing varying concentrations of LiCl. (a) Cell survival. Cells were stained with Trypan blue solution (0.4%). (b) Lactate dehydrogenase (LDH) enzyme activity. (c) Cell migration (quantified by Image J). (d) ALP staining (evaluated by Image J) (e) Absorbance of dissolved crystals after staining with Alizarin Red S. (n=3, One-way ANOVA with Tukey's Kramer post hoc, significance was indicated by different letters a, b, c and d).

⑤ Alizarin red staining

The absorbance from the solutions of the dissolved crystals from cells cultured in the presence of S-PRG/LiCl-100 mM eluates as compared with the other groups (Fig. 2e).

3. Immunofluorescence staining

The S-PRG/LiCl-100 mM treatment led to a strong expression of β-catenin in the odontoblastic cell layer, distributed in a localized pattern at 7 days. In day-14 samples, a lower expression of β-catenin was observed with S-PRG/LiCl-100 mM treatment, similar to that of the intact tooth (Fig. 3). The expression of Axin2 in the odontoblastic cell layer of the S-PRG group was

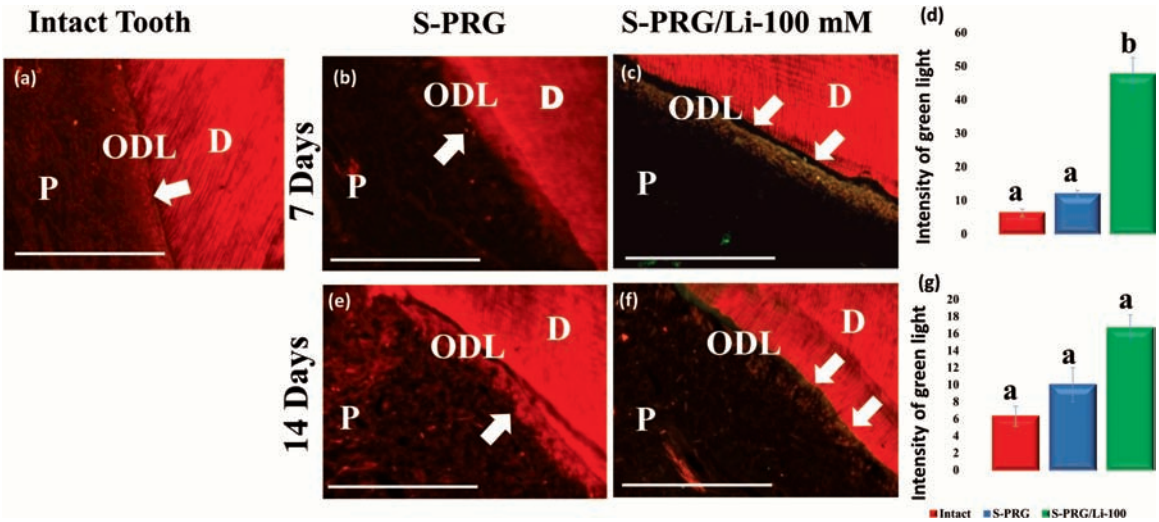

Fig. 3. β-catenin expression. (3a-c, e and f) Micrographs show β-catenin expression in the odontoblastic cell layer in the normal tooth structure and after capping with S-PRG and S-PRG/Li-100 mM at 7-and 14-days post-operatively. (3d and g) Quantitated values of β-catenin expression in three randomly selected samples from each group, (n=3), One-way ANOVA with Tukey's Kramer post hoc, significance indicated by different letters a and b). (P: pulp, ODL: odontoblastic cell layer, D: dentin, Scale bar =100 μm).

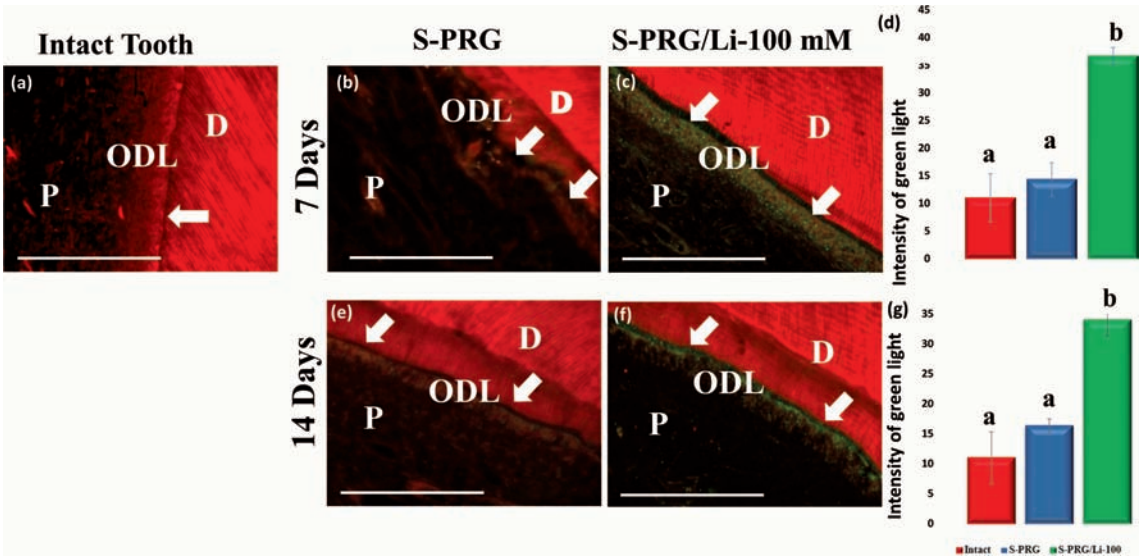

Fig. 4. Axin2 expression in the odontoblastic cell layer in the intact control, S-PRG, and S-PRG/Li-100 mM groups at 7 and 14 days, postoperatively (4a-c, e and f). (4d and g) Quantitated values of Axin2 expression, as determined using BZX-8000 view analyzer software, (n=3). One-way ANOVA with Tukey's Kramer post hoc, significance indicated by different letters a and b. (P: pulp, ODL: odontoblastic cell layer, D: dentin. Scale bar =100 μm).

similar to that of the intact control group at day 7 and day 14. However, treatment with S-PRG/Li-100 mM led to strong expression of Axin2 in the odontoblastic cell layer at both time points (Fig. 4).

The Axin2 expression in higher-magnification images showed diffuse pattern of expression indicated a cytoplasmic location of Axin2, while β-catenin demonstrated a nuclear localization.

4. Lithium levels in the serum

The concentration of lithium ions in the peripheral blood circulation was less than 0.01 mmol/L at 24 h after the topical application of S-PRG/LiCl-100 mM cement.

Discussion

In the current study, we enhanced the efficacy of S-PRG fillers as dental pulp-capping agents by incorporating lithium ions. This novel combination activates the Wnt/*β*-catenin signaling pathway as a part of the healing process of the dentin-pulp complex. The pulp capping experiments resulted in complete dentin formation when the S-PRG fillers were combined with lower concentrations of LiCl (Fig. 1). In vitro studies were also performed to evaluate the behavior of hDPSCs following treatment with eluates of the experimental cements bearing different Li concentrations. We found that eluates from S-PRG/Li-100 mM samples could significantly enhance cell migration, differentiation, mineralization with the best biocompatibility as compared with the other groups (Fig. 2b, d and e). In contrast S-PRG/Li-1000 mM decreased cell profiles, with fewer surviving cells and the highest expression of LDH. The *in vitro* results of this study may help to explain the pattern of tertiary dentin formation in previous pulp-capping trials.

The mechanical properties of the cements were also evaluated. No statistical difference was observed in the shear bond strength to the dentin after LiCl incorporation (Table 2). However, S-PRG/Li-100 mM and S-PRG/Li-1000 mM specimens had significantly decreased the compressive strength compared to other groups. A similar tendency was reported by Vahabzadeh et al.[29] They noticed deterioration in the mechanical characteristics of *β*-Tri calcium phosphate cement when doped into higher concentrations of pure lithium oxides.

Wnt/*β*-catenin signaling pathway when activates, *β*-catenin molecules accumulate in the cytoplasm of cells and then translocate into the nucleus to trigger the expression of different genes associated with tooth development at various stages (Fig.5). In the current study, LiCl-100 mM was shown to activate this signaling pathway. As suggested by Jope et al.[30], this may be achieved by inhibiting GSK-3*β* molecules that deregulate the function of the destruction complex and produce signals. Immunofluorescence-stained sections showed intense *β*-catenin expression in the odontoblastic cell layer at day 7 in samples treated with S-PRG/Li-100 mM (Fig. 3). The magnified images further suggested translocation of *β*-catenin into the nuclei of these odontoblastic cells, indicative of canonical signal activation. *β*-catenin expression in the S-PRG/Li-100 mM group declined at the later time point, suggestive of a temporary activation of Wnt/*β*-catenin signaling as part of the reparative process. The outcomes of this assay are in agreement with the study by Han and colleagues[31].

On the other hand, Axin2 was highly expressed in

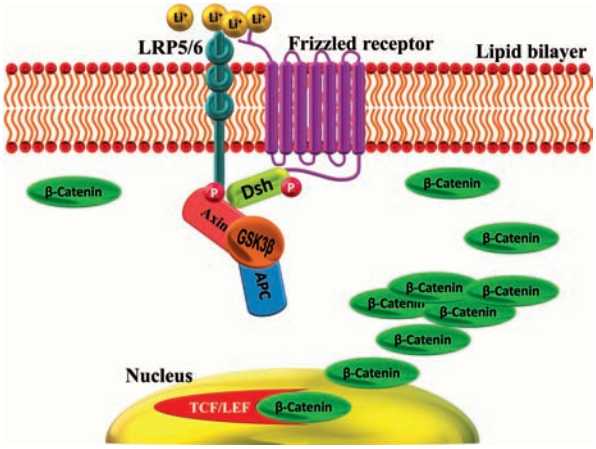

Fig. 5. Wnt/*β*-catenin signaling pathway. Starts when the 7-transmembrane frizzled receptor binds to lithium-ions that released from S-PRG cements which provides a binding site for Low density Lipoprotein receptor to bind axin2 and phosphorylate the Dishevelled protein. The activation of dishevelled protein abolish the action of beta catenin destruction complex because it allows the lithium binds to GSK-3*β* leading to hypo phosphorylation of *β*-Catenin. Then Hypophosphorylated *β*-catenin accumulate in the cytoplasm and starts to translocate into the nuclei where they form a complex with T- Cell Factor/ Lymphoid Enhancer Factor and transcribed into the target genes to achieve different cell functions.

the S-PRG/Li-100 mM pulp-capped groups at 7 and 14 days as compared with S-PRG cement without lithium or the intact teeth. Babb et al. previously reported that Axin2 has roles in β-catenin deregulation which may or may not involve other signaling molecules[32]. We found that even the S-PRG/Li-100 mM sample released low concentrations of lithium ions (<0.01 mmol/L) into the peripheral circulation. This level is lower than that described by Hanak et al.[33]. Hanak and colleagues conducted tests to ascertain the level at which the administration of Li_2CO_3 by intraperitoneal injection caused acute poisoning. In their study, the lithium concentration in the plasma was higher than 1.0 mmol/L.

Conclusions

S-PRG/Li-100 mM showed high biocompatibility, and promoted the biological functions of hDPSCs *in vitro*, and induced reparative dentin formation in biomimicry approaches using rat models. These effects were due in part to the activation of Wnt/β-catenin canonical signaling. Overall, this novel cement is suitable and recommended for clinical implementation as a direct pulp-capping cement.

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